Novel strategy to inhibit the oncogenic transcription factor c-Myc

in triple-negative breast cancer

Rita Mejzini

(31695202)

Thesis submitted in fulfilment of a Bachelor of Science with Honours in

Biomedical Science

Murdoch University

School of Veterinary and Life Sciences

Supervisor: Associate Professor Pilar Blancafort

Harry Perkins Institute of Medical Research

2016
Acknowledgments

Firstly, I would like to thank my supervisor Pilar Blancafort for her support and advice throughout the year and for this opportunity to learn new skills and put them into practice. I would also like to thank my teammates Ciara Duffy, Edina Wang, Anabel Sorolla and Agustin Sgro for their guidance, support and encouragement which was much needed and appreciated. I would also like to acknowledge the Harry Perkins Institute and all its supporters and staff. It was a wonderful place to carry out my research with excellent facilities.

This research was supported by grants from the Australian National Health & Medical Research Council and The Cancer Council of Western Australia. The 1746 Phylomer and the 1746-Omomyc and Omomyc peptide were produced and provided by collaborators, Phylogica Ltd.

All work was my own apart from some of the dose response data for the CellTiter-Glo Assays for 1746-Omomyc which had previously been carried out and was contributed by PhD student Edina Wang.

Declaration

I declare this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution.

Rita Mejzini
Abstract

Triple-negative breast cancer (TNBC), characterised by the minimal expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2), is an aggressive clinical phenotype. Its expression profile means it is not amenable to hormone therapy or HER-2 directed treatments with systemic treatment options limited to cytotoxic chemotherapy. There is therefore an unmet need for new targeted treatments for this cancer subtype. Transcription factor c-Myc (Myc) is frequently deregulated in these cases and is implicated in breast cancer development and progression. Omomyc is a peptide able to interfere with the protein-protein interactions of Myc, inhibiting transcriptional activation of its target genes. Here I report on the use of a new cell penetrating peptide (CPP), 1746, to deliver Omomyc to TNBC cells and on a truncated version of the Omomyc peptide (Omi) linked to 1746 (1746-Omi) and the traditional CPP Penetratin (Penetratin-Omi). I found 1746-Omomyc reduced the viability of TNBC cells overexpressing Myc by reducing proliferation. The truncated peptides were not as potent but demonstrated greater selectivity towards cancer cells. Penetratin-Omi had a greater activity than 1746-Omi and reduced cell viability primarily through cell death rather than reduced proliferation. The effect of 1746-Omomyc and Penetratin-Omi on the regulation of selected downstream Myc targets was determined by qRT-PCR and was consistent with but does not confirm Myc inhibition. This demonstrates the potential of 1746-Omomyc as an effective Myc inhibitor in TNBC. The shortening of Omomyc’s sequence reduced the peptides activity with much higher concentrations of 1746-Omi and Penetratin-Omi required to effect cancer cell viability. The mechanism of action of Penetratin-Omi also differed from 1746-Omomyc, indicating the possibility of a cargo-dependent cytotoxicity at high concentrations. Improving the design of the peptides could increase efficiency and reduce the possibility of any cytotoxic side effects.
1. Introduction

Triple-negative breast cancers (TNBCs) are those lacking overexpression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2) [1]. These are three of the most common receptors known to fuel breast cancer growth and are the targets of hormone and drug therapies. With no effective targeted treatment available, systemic treatment for patients with TNBC is limited to chemotherapeutical approaches which lack selectivity and often have undesirable side effects. Non-targeted chemotherapy drugs may also be limited in their ability to penetrate tumours, leading to drug resistance [2]. These aggressive malignancies are associated with resistance to chemotherapy, metastases, high rates of recurrence and mortality [1]. There is thus a great need for new targeted therapies to treat this breast cancer subtype.

The majority of TNBCs are classified as basal-like tumours, which are associated with overexpression and copy number amplification of the oncogenic transcription factor \( c\text{-Myc} \) \((Myc)\) [3]. Myc is a non-redundant driver of cancer proliferation and is involved in, angiogenesis, cellular metabolism and resistance to apoptosis [4]. Transcription factors are not easily targeted with conventional drugs as they lack small molecule binding pockets. However, they often have highly conserved protein-protein interactions with binding partners and co-factors, which are essential for transcriptional activity [5]. Targeted interference with the protein-protein interactions of oncogenic transcription factors such as Myc may be a useful strategy to inhibit cancers such as TNBCs that are refractory to current treatments.

Omomyc is a protein designed to interfere selectively with the protein-protein interactions of Myc and therefore inhibit transactivation of its target genes [6]. Promisingly, this
protein has demonstrated the ability to eradicate cancer in several animal models [7, 8]. There is also evidence that the effects of Omomyc induced Myc inhibition in normal cells are mild and reversible [9]. Omomyc shows great potential as an anti-cancer agent. On its own, however, it lacks the ability to penetrate cells. Cellular and nuclear penetration are essential for the protein to reach its targets. Conjugation of Omomyc to a Cell Penetrating Peptide (CPP) could be a useful strategy to aid in its internalisation as CPPs are able to mediate the internalisation of molecules with low permeability into cells. Another barrier to the potential therapeutic usefulness of the Omomyc protein is its large size. It may be beneficial to produce a truncated version of the protein. This would open up the technological possibilities for its production and modification with potential benefits in cellular penetration, product purity and reduced manufacturing costs.
2. Literature Review

2.1 Triple-Negative Breast Cancer

2.1.1 Overview

Breast cancer is the most common cancer in women worldwide with over 1.6 million new cases diagnosed each year [10]. The prevalence of triple-negative breast cancers can vary in different populations but are reported to account for 15 to 20% of all breast cancer cases [11]. Gene expression profiling studies have identified at least six intrinsic molecular subtypes of breast cancer including luminal A, luminal B, normal breast-like, HER2 enriched, claudin-low and basal-like breast cancers [12]. The majority of TNBCs are of the basal-like subtype [13]. Basal-like breast cancers are a heterogeneous group that express genes and proteins usually found in basal or myoepithelial cells of the normal breast tissue [14]. They are associated with an aggressive phenotype, high histological grade, poor patient prognosis, and high rates of recurrence and metastasis [15]. Tumours of the basal-like subtype have more frequently overexpress Myc in comparison to the other subtypes [3, 16]. This overexpression has been shown to promote changes in cellular proliferation, differentiation, cell cycle progression, growth, metabolism, DNA replication, cell adhesion and metastasis [17].

As well as basal-like tumours, a substantial number of TNBCs have also been found to be of the more recently discovered claudin-low intrinsic subtype of breast cancer [18]. Claudin-low tumours are more enriched in epithelial-to-mesenchymal transition features, immune system responses, and stem-cell associated biological processes [19]. They are the most undifferentiated tumours along the mammary epithelial hierarchy. Tumours of the claudin-low subtype are reported to have lower expression of Myc than basal-like tumours, but higher expression of epidermal growth factor receptor (EGFR) and other proliferation-related gene expression signatures [20].
2.1.2 Treatment and Research for TNBC

Due to the absence of ER, PR and HER-2 overexpression in TNBCs, hormone therapy and HER-2 directed therapies are not useful treatment options for patients with these tumours. Systemic treatment is therefore limited to cytotoxic chemotherapy. Although TNBCs have a good initial response to chemotherapy, an early complete response does not correlate with overall survival as the risk of relapse in the first 3 to 5 years is significantly higher than with hormone receptor positive types of breast cancer [21]. For patients with residual cancer post chemotherapy, those with TNBCs also had a worse prognosis than those with the other subtypes [22]. It is of interest that tumour cells that do survive chemotherapy show features similar to the claudin-low subtype of breast cancer [23].

Several strategies to treat TNBC which aim to inhibit different targets or processes are being investigated. Currently, the key strategy is the development of Poly (ADP-Ribose) Polymerase (PARP) inhibitors designed to target cells with defective DNA repair mechanisms. Several PARP Inhibitors are currently being evaluated in Phase I or II clinical trials as a single agent or more commonly in combination therapies, with the aim of increasing sensitivity to chemotherapy or radiotherapy [24]. Other strategies have included the use of angiogenesis inhibitors such as Bevacizumab, a monoclonal antibody directed against vascular endothelial growth factor. Bevacizumab is used in combination therapies for those with metastatic HER-2 negative breast cancers but has not been shown to increase overall survival in Phase III clinical trials [25]. EGFR inhibitors such as Cetuximab have also been trialled in combination therapies for the treatment of TNBC but have again shown only modest results [26, 27]. Another approach has been the use of SRC-family kinase inhibitors such as Dasatinib. This was also shown to have a very limited activity in patients with TNBC [28]. mTOR inhibitors, such as Everolimus, have
also been trialled in combination therapies for patients with TNBC. Everolimus did not improve clinical response rates, however, and was associated with more adverse events [29]. It is clear there is still an unmet need to develop effective targeted treatments for patients with TNBC. Myc’s non-redundant role in cancer proliferation and its associations with the TNBC subtype makes inhibition of this oncogenic transcription factor a promising strategy.

2.2 What is Myc?

2.2.1 The Role of Myc

The Myc protein has been shown through microarray studies to directly or indirectly influence the expression of up to 15% of all genes [30]. It is a key regulator involved in controlling a large range of cellular processes including cell growth and division, apoptosis, cellular differentiation, angiogenesis, cell adhesion, cellular metabolism and motility [30]. Myc levels are low in quiescent cells, with levels rapidly increasing upon entry into the cell cycle and then declining to remain at a basal level in cycling cells [31].

Myc drives transcription via the recruitment of co-factors to target gene promoters [32]. It is involved in cellular proliferation and controls the progression from the G1 to S-phase of the cell cycle via activation of downstream targets such as cyclin E/Cdk2, and repressing cyclin-dependent kinase (Cdk) inhibitor p21 [33, 34]. Cellular proliferation is also facilitated by Myc’s activation of cyclin D1, Cdk4, Cdc25A, E2F1 and E2F2 [35]. Paradoxically, as well as promoting proliferation, Myc also acts as a tumour suppressor, with high levels of Myc sensitising cells to a range of pro-apoptotic stimuli [36]. A significant portion of Myc’s activity is cell type specific. However, a core set of target genes that are cell-type independent have been identified and are dominated by genes
involved in biomass accumulation through ribosome biogenesis and RNA processing [37].

Myc has also been found to play an important role in stem cell biology and is involved in maintaining pluripotency and self-renewal in embryonic stem cells [38]. In 2006 it was named as one of the four Yamanaka factors needed to produce induced pluripotent stem cells (iPSCs) [39]. Since that time Myc has been found to be non-essential for iPSC production, although its inclusion greatly improves the efficiency [40].

2.2.2 Structure and Mechanism of action of the Myc Protein
The Myc protein contains a transcriptional activation domain at its amino-terminus and a 100 amino acid basic helix-loop-helix-leucine zipper (bHLHZ) region at its carboxy terminus. The bHLHZ specifies dimerization with another bHLHZ protein, Max, which is required to activate transcription. Myc-Max heterodimers recognise and bind to DNA at E-box elements (CACGTG) via interactions between the basic regions of the bHLHZs and the major groove in the DNA, activating transcription at promoters containing these elements [41]. Myc-Max dimers can also act as repressors of Myc-regulated genes through indirect recruitment to DNA via the zinc-finger protein Miz-1 [30]. Another class of bHLHZ proteins including Mad1 (Mad) are also able to heterodimerize with Max and bind to E-box elements. The Mad-Max heterodimer works in opposition to Myc-Max and acts as a transcriptional repressor, inhibiting cell growth and decreasing cellular proliferation [42]. It appears that the fate of a cell to proliferate/transform or differentiate/become quiescent is affected by competition between Myc-Max and Mad-Max heterodimers for common DNA targets.
2.2.3 Regulation of Myc

Although regulated at several levels, primarily regulation of Myc is at the level of transcription in response to growth factor signalling [43]. EGFR is involved in this regulation via activation of pathways which drive cellular proliferation (ERK pathway) and provide protection from Myc-mediated apoptosis (PI3K-Akt pathway) [44, 45]. Overexpression of EGFR is common in TNBC, with 84% of basal-like tumours exhibiting this feature [46]. The EGFR signal cascade is implicated in cellular proliferation, angiogenesis, metastatic spread and the inhibition of apoptosis [47]. It is estimated that EGFR is likely a substitute for the major proliferation pathways of breast cancer induced by activation of HER-2, ER and PR proteins which are not highly expressed in TNBC [47].

Interestingly, breast cancer 1 (BRCA1) is also directly involved in the regulation of Myc and acts as a repressor, binding to Myc and inhibiting its transcriptional and transforming
activity [48]. BRCA1 mutations are common in TNBCs and the clinical and pathologic features of basal-like breast cancer overlap with hereditary BRCA1 related breast cancer [11]. Mutations in the BRCA1 gene predisposes patients to develop many cancers as BRCA1 is involved in multiple cellular processes including DNA repair, cell cycle control, apoptosis and transcriptional regulation [49]. It is not surprising then that Myc amplification is a frequent event in tumours with BRCA1 inactivation [50].

Myc is also regulated at several other levels. The export of the Myc RNA into the cytoplasm and its subsequent translation are tightly controlled. Post-translational modifications and interactions with other proteins also control Myc’s activity [17]. Furthermore, the effects of Myc can be finely tuned to the state of the cell. For example, it preferentially binds chromatin in certain states and can preferentially associate with E-boxes containing epigenetically inherited markers [51]. Every population of cancer cells could therefore potentially have different epigenetic patterns that dictate Myc’s binding. As Myc transactivation is explored, new levels of complexity continue to make resolving Myc’s function a challenge. Although it influences a large number of target genes, its effect on any given gene remains weak with the expression of the majority of target genes being amplified only two-fold [52].

2.2.4 Deregulation of Myc in Cancer

Increased expression of Myc is thought to occur in an average of 50% of human cancers [17]. It is seen in a diverse range of tumour types including a significant proportion of lymphoma, melanoma, multiple myelomas, neuroblastoma, ovarian, prostate as well as colon, lung and breast cancers [53]. Furthermore, Myc has been shown to be a non-redundant driver of tumorigenesis. For example, studies involving acute activation of
Myc in a reversibly switchable transgenic mouse model have demonstrated that sustained Myc activity is required for tumour maintenance [54].

Several mechanisms may lead to increased expression of Myc. Unlike other oncoproteins such as Ras, there need not be any changes to the coding sequence of Myc for it to unleash its oncogenic potential. In most Myc-driven cancers it is a change to another locus (such as BRCA1) that causes a downstream effect able to disturb critical regulatory mechanisms of Myc, leading to Myc overexpression, enhanced translation or increased protein stability [16, 55].

The levels of Myc present in the cell govern how it interacts with the genome. In cells expressing high levels of Myc, it can accumulate at the promoter region of active genes leading to transcriptional amplification and a decoupling of cellular proliferation from growth-factor stimulation. This uncontrolled cellular proliferation can result in genomic instability, escape from immune surveillance and immortalization [56].

Figure 2: Cellular processes controlled by Myc during normal conditions (left) and tumorigenesis (right) [53].
Myc’s involvement in tumorigenesis also occurs via several other mechanisms. Telomerase, a protein involved in maintaining telomere length, is directly activated by Myc through induced expression of its catalytic subunit, telomerase reverse transcriptase (TERT). Constitutive expression of Myc and TERT can induce cell immortalisation [57]. Myc is also essential for angiogenesis during tumour development and progression and is involved in controlling vascular endothelial growth factor expression levels [58]. Myc’s reach in cancer development also extends to metastasis through activation of miR-9, a negative regulator of the metastasis suppressor E-cadherin, leading to increased cellular motility and invasiveness [59].

### 2.3 Strategies for Myc Inhibition

Myc’s activation in a wide variety of cancers and its involvement in proliferation and apoptosis have made it an attractive target for potential new cancer therapies. Transcription factors have traditionally been thought to be undruggable due to their lack of enzymatic activity and typical topography, which is large and devoid of features such as small molecule binding sites. Recently however, strategies have been devised that target Myc at all levels. This includes those aimed at inhibiting Myc’s transcription or translation, disrupting Myc/Max dimerization, blocking Myc’s interactions with co-factors, interfering with the Myc/Max homodimer interaction with DNA, inhibiting expression of Myc target genes and promoting Myc protein degradation in the cell [53]. These strategies usually involve the use of small interfering RNA (siRNA), antisense oligonucleotides or small molecules. Protein-based solutions are also being developed.
2.3.1 siRNA

siRNA is able to induce enzymatic degradation of any mRNA complementary to it through interactions with the RNA-induced silencing complex (RISC) [60]. This has been utilised to target *Myc* mRNA sequences with some success. For example, siRNA expressed from a plasmid vector has successfully targeted *Myc* mRNA, reducing *Myc* expression and suppressing breast tumour growth in mice [61]. However, there have been several impediments to this approach preventing the translation into new therapies. The main obstacle to the use of siRNA as a therapeutic is rapid siRNA degradation in the bloodstream by nucleases, resulting in a half-life of only a few minutes [62]. When siRNA is delivered systemically, uptake into target organs and cells is generally poor [63]. There has been more success with siRNA when delivered locally in non-cancerous disease models into tissues such as the eye or lung [64, 65]. The ability to overcome this problem through chemical modifications is restricted as limited modifications can be introduced for the molecule to remain functional within the RISC [66]. Efficient delivery of siRNA remains the most challenging hurdle in the development of siRNA as a therapeutic platform. Strategies under investigation to overcome this include creating siRNA conjugates, antibody complexed siRNAs and the use of liposomes and lipoplexes [60].

There have been other challenges to the therapeutic use of siRNA which have hampered progress. These include its ability to activate the innate immune system in mammals and issues with off-target effects which have been shown to vary depending on the transfection method and the mRNA expression profile of the target cell [67, 68].

2.3.2 Antisense Oligonucleotides

Another approach to inhibiting Myc has been antisense oligonucleotides. These are single-stranded DNA molecules specifically targeted to hybridize and inhibit a particular
mRNA. This also results in the degradation of the target mRNA, although this time through recruitment of Ribonuclease H (RNase H). Chemical modifications do not seem to affect RNase H activity as readily as seen with the RISC. A typical antisense oligonucleotide drug therefore includes a phosphorothioate backbone that links the nucleosides and modified nucleotides at each flank to protect it from exonucleases and increase stability in vivo [69]. Antisense oligonucleotides have been used successfully to reduce Myc expression and cellular proliferation of breast cancer cell lines and in animal models of Burkitt's lymphoma, although these have not yet translated into new therapies [70, 71]. One problem slowing the development of phosphorothioate antisense oligonucleotide drugs has been that some sequences allow interactions with Toll-like receptors, inducing a strong immunostimulatory response [72]. The backbone has also imparted a toxicity profile that varies with different sequences and can lead to increased coagulation time, pro-inflammatory effects and renal tubule changes [69]. Attempts have been made to bring antisense oligonucleotide based cancer drugs to market. For example, Oblimersen, which targets the mRNA of the gene encoding B-cell lymphoma 2, has been tested in over 40 clinical trials since 1999 but the Oblimersen programme was terminated after reports of only modest effects, no effects, or missing the expected primary targets [73]. Other oligo-based approaches to Myc inhibition under investigation include the use of triple helix and tetraplex forming oligos to inhibit Myc mRNA expression, and phosphorodiamidate morpholino oligomers to attack translation by preventing ribosomal assembly [74-76].

2.3.3 Small Molecules
The use of small molecules to target transcription factors is also under investigation. Small organic molecule are generally defined as those with molecular weights below 900
Daltons. Inhibition can be aimed at several levels including blocking transcription or translation, promoting protein degradation, interfering with interactions with co-factors or indirectly inhibiting the function of key target genes [53]. The primary strategy in the case of Myc has been to disrupt the protein-protein interaction of the Myc-Max dimer. The selective disruption of protein-protein interactions by small molecules has been a long sought after goal. There are however a number of factors that have hampered the successful development of interfering small molecules. Unlike enzyme inhibitors, which compete with natural substrates for small pockets shielded from the aqueous environment, protein-protein interaction inhibitors aim to disrupt interactions that occur over large surfaces. These surfaces are associated with high free energies of association and are generally devoid of regions that readily accommodate small molecules [77]. Small molecules able to inhibit heterodimerization of Myc and Max were traditionally identified via the screening of large peptidomimetic libraries [78-80]. Yin et al. utilised a yeast two-hybrid-based assay to screen large numbers of small molecules and identified compounds 10058-F4 and 10074-G5, which showed specificity for disrupting the interaction of the Myc-Max heterodimer [81]. Further development of these two molecules led to studies in mice. However, a lack of antitumour activity was seen due to rapid metabolism of these molecules [82, 83]. Direct small molecule inhibitors of Myc have had limited success in vivo to date due to short half-lives, rapid metabolism, efflux from target cells or inefficient tumour penetration [83, 84]. Work is continuing to improve their in vivo stability.

2.3.4 Proteins
An alternative strategy to inhibit Myc-Max dimerization is the use of a protein rather than an oligo-based or small molecule approach. There are several advantages to using a protein. They generally display a very high affinity to interact with their target, offering
greater efficacy, selectivity and specificity than small organic molecules [85]. Another advantage is the typically low toxicity profile of protein-based drugs. This is partly because the degradation products of proteins are amino acids, minimising the risks of systemic toxicity [86].

Proteins have traditionally been thought of as poor drug candidates. This is usually for reasons involving in vitro and in vivo instability or issues related to poor cellular penetration or delivery. Protein drugs generally have a low oral bioavailability due to their rapid degradation by the proteases in the digestive system, usually requiring injection. Bioavailability may also be reduced by their susceptibility to proteolytic degradation and hydrolysis in plasma or hepatic or renal clearance [87]. Issues with low permeability across biological membranes also need to be overcome. External factors such as temperature, pH, contaminants or impurities can compromise the chemical and physical stability of a protein. A thorough understanding of their biological and physiochemical characteristics is therefore required to ensure the efficacy and safety of any protein based drug [88].

In recent years, however, the interest in protein-based drugs has increased. The global market for therapeutic proteins has been growing at a moderate rate and is forecast to reach $141.5 billion in 2017 [89]. Several strategies have been devised to overcome the issues with stability and delivery. For example, proteins can be chemically modified by altering one or more amino acids to create a protein analogue with optimised pharmacokinetic properties. This strategy was successful in the development of a monomeric rapidly acting insulin analogue [90]. Other methods involve acylation to improve circulation time in the blood or PEGylation to reduce enzymatic degradation and
improve receptor-mediated uptake [91, 92]. The advantages and disadvantages of each of the strategies for Myc inhibition are summarised in Table 1.

Table 1: Advantages and disadvantages of the main strategies for Myc inhibition

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>siRNA</td>
<td>High specificity</td>
<td>Poor stability in plasma</td>
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<td>Poor intracellular uptake</td>
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<td>Limited chemical modifications</td>
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<td>Immunogenicity</td>
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<td>Off-target effects</td>
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<tr>
<td>Antisense oligonucleotides</td>
<td>High specificity</td>
<td>Poor intracellular uptake</td>
<td>[69, 72]</td>
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<td></td>
<td></td>
<td>Chemistry dependent toxicities</td>
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<td>Small molecules</td>
<td>Greater bioavailability</td>
<td>Short half-lives</td>
<td>[82-84]</td>
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<td></td>
<td>Lower production costs</td>
<td>Rapid metabolism</td>
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<td></td>
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<td>Efflux from target cells</td>
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<td></td>
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<td>Inefficient tumour penetration</td>
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<tr>
<td>Proteins/Peptides</td>
<td>High affinity to target</td>
<td>Low oral bioavailability</td>
<td>[85, 87, 88]</td>
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<td></td>
<td>High selectivity and specificity</td>
<td>Proteolytic degradation</td>
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<td>Low toxicity</td>
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Improvements have also been made in protein drug delivery with various nanoparticulate, protein and peptide delivery systems being developed [93]. These improvements make protein-based drugs an attractive option especially in situations where small molecules may not be suitable, such as the targeting of transcription factors.

2.4 Omomyc

2.4.1 What is Omomyc?

Omomyc is a 92 amino acid protein able to specifically inhibit the dimerization of Myc and Max. Omomyc was constructed by Soucek et al. who utilised the fact that Myc is unable to form homodimers at physiological concentrations [6]. Four charged amino acids two glutamates (E57, E64) and two arginines (R70, R71), located in the leucine zipper were found to display steric and electrostatic clashes preventing homodimers of Myc
forming. Omomyc’s sequence was taken from the bHLHZ domain of Myc with the critical amino acids preventing dimerization substituted. This altered the dimerization specificity of Omomyc allowing it to homodimerize as well as form heterodimers with wild-type Myc and Max. The first glutamate was substituted with threonine and the other changes mimicked the corresponding amino acids in the Max protein. In the-Max homodimer, glutamine and asparagine residues at positions 70 and 71 of the two monomers form a remarkably stable tetrad (QN/QN) of major importance for structure stabilisation. Poor Myc homodimerization arises due to the disruption of this tetrad, since positions 70 and 71 in Myc are occupied by amino acids with the same polarity. The presence of three charged residues (the two glutamate 57, 64 and arginine 71) at three consecutive positions also destabilise the zipper region [6]. Amino acid sequences can be seen in Table 2.

**Table 2: Partial Amino Acid sequences of Myc, Max and Omomyc (numbers refer to amino acid position in Omomyc)**

<table>
<thead>
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<th>Myc</th>
<th>Omomyc</th>
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Omomyc can suppress the transcription of Myc target genes via several mechanisms. Firstly, by sequestering Myc in the Omomyc-Myc heterodimers, which are incapable of binding to E-boxes, less Myc is then available to form the active Myc-Max heterodimers. Secondly, by forming Omomyc-Max heterodimers, which can bind to E-box elements and act as a competitive inhibitor of the Myc-Max dimer for these sites. Omomyc is also able to form stable homodimers which also compete with Myc-Max dimers for DNA binding sites [94]. Whilst Omomyc is capable of inhibiting transcriptional activation by Myc, it has been found to enhance Myc-induced repression and apoptosis [95]. This is
because unlike transactivation, repression by Myc is not dependent on interactions with E-box elements but rather with proteins such as Miz1, which is able to interact with Omomyc and other DNA elements [96]. Omomyc does not, therefore, cause a global inhibition of Myc function, but rather channels its activity towards transrepression; a feature which may contribute to its success as an anti-cancer agent. Omomyc has been used successfully in animal models and has been able to eradicate KRAS-driven lung cancer, inhibit Myc-induced papillomatosis and been effective against glioma in mice [97-99].

2.4.2 Effects of Omomyc in Normal Cells

Myc is a factor in the coordination of a diverse range of processes necessary for the normal growth and expansion of somatic cells, prompting concerns that Myc inhibition may cause serious side effects in normal tissue. As Myc is expressed at low to undetectable levels in most tissues and organs due to their quiescent nature, there is potentially a relatively large therapeutic window in which to target aberrantly expressed Myc in tumour cells. The exception to this would be highly proliferative cellular populations such as in the haematopoietic system and gastrointestinal precursor cells. Based on this, side effects would be expected to be similar to those following treatment with cytotoxic agents that non-specifically target all proliferating cells, such as loss of intestinal villus integrity and bone marrow aplasia. The effect of Myc inhibition via Omomyc was evaluated in a study on transgenic mice with inducible Omomyc expression [9]. After four weeks of Omomyc induced Myc inhibition, the animals showed no discernible histological changes in any slowly proliferating tissues or organs including the heart, lung or kidney. As expected, tissues that experience rapid cell turnover were affected. The basal layer of the skin epidermis exhibited a marked reduction in
proliferating cells and thinning. Hair regrowth was inhibited and there was a significant decrease in proliferation in the intestinal crypts and attrition of villi in the small intestines. Loss of spermatogonia and spermatocytes was also evident. However, after withdrawal of the stimulus inducing Omomyc expression, all these effects were reversed within one week with no discernible deficits or pathology found in any tissue. This study also evaluated the effect of Myc inhibition on the haematopoietic lineages. They reported significant inhibition of proliferation in bone marrow and rapid onset of anaemia and leucopenia. However, by two weeks of sustained Myc inhibition, blood counts had returned close to normal levels. Throughout these tests there were no signs of induced apoptosis, aberrant differentiation or loss of tissue integrity and no signs of ill health or distress were seen in any of the animals. Omomyc was expressed in all tissues in this model. In carrying over to a therapeutic setting, targeting of Omomyc to cancer cells could significantly reduce these side effects. This could be achieved via several methods, for example, by conjugation to a sequence that targets integrins or receptors overexpressed on the cancer cells of interest or through the use of targeted nanoparticles.

2.4.3 Omomyc Delivery

Omomyc has shown some encouraging results as a potential therapeutic for cancer treatment. A challenge to the success of this peptide as a useful anti-cancer agent is how it is directed into the cancer cells. Some investigative studies into the potential of Omomyc in cancer cell lines thus far have used retroviral vectors to have Omomyc produced endogenously [96, 98]. Mouse models have utilised transgenic mice in which Omomyc expression can be systemically but reversibly induced by administration of doxycycline [97, 99]. Whilst these methods are useful in the lab, alternative approaches are needed to proceed to the clinic. One possibility is the use of an adenoviral vector.
Adenovirus particles can infect a host cell and release its genetic transcript into the nucleus to be transcribed into a protein product. This approach is becoming more popular in gene therapy research. Adenovirus vectors have been used to mediate expression of Omomyc in lung cancer cells [100]. Although anticancer activity has been established via the use of adenoviral vectors in preclinical studies, subclinical use of therapeutic adenoviral vectors has been limited due to several obstacles including clearance by the immune system and limitations in tumour cell delivery [101]. There is thus a need to investigate alternative strategies to deliver Omomyc and other anticancer therapeutics to cancer cells.

2.5 Cell Penetrating Peptides for the Delivery of Omomyc

2.5.1 What are Cell Penetrating Peptides?
The acceleration in the production of large therapeutic molecules in recent years has increased the demand for new drug delivery systems able to circumvent the issues regarding in vivo stability, cellular uptake, and bioavailability that many of these larger molecules display. Ideally, these drug delivery systems will be able to deliver their cargo efficiently to the target cells at low doses, lack toxicity, display rapid endosomal release and facilitate the therapeutic application of the cargo. Several non-viral strategies for drug delivery are being investigated including nanoparticles, lipid and peptide-based solutions.

One peptide-based approach that is gaining momentum is the use of cell-penetrating peptides. CPPs (also known as protein transduction domains) are a class of peptides able to mediate the internalisation of themselves and a non-permeable molecule (usually conjugated to the CPP) into a cell. CPPs are generally less than 30 amino acids in length and are usually derived from natural protein sequences. The first CPPs (Tat and
Penetratin) were discovered in the late eighties and early nineties. Tat was derived from the HIV-1 Trans-Activator of Transcription (Tat) protein after the observation was made that this protein was able to enter cells independently and translocate to the nucleus [102, 103]. Penetratin was developed shortly after and corresponds to a 16 amino acid sequence found in a transcription factor, Antennapedia (Antp), in Drosophila melanogaster. It was derived after the discovery that this protein could be secreted without a signal peptide and that its subsequent uptake by neighbouring cells was receptor-independent [104]. Other CPPs have since been developed such as the VP22 peptide from the herpes simplex virus, and the chimeric transportan peptide. Tat and Penetratin, however, remain the most extensively characterised CPPs.

The interest in CPPs has been primarily due to their low cytotoxicity and their ability to deliver a diverse range of cargo into cells. Although they are able to aid in the internalisation of various biomolecules including small molecules, oligonucleotides, plasmid DNA and even liposomes and nanoparticles, a significant focus has been on mediating the delivery of peptides and proteins [105]. CPPs can be classed into two broad groups; those that require the cargo to be chemically linked and those able to form stable non-covalent complexes with the cargo. They can also be grouped based on how amphipathic they are.

Due to technical artifacts, the mechanism of cellular uptake of CPPs remained puzzling for a long while. It was eventually discovered and confirmed that uptake of many CPPs is mediated by endocytosis [106, 107]. CPPs such as Tat and Penetratin interact with the surface of a cell through electrostatic binding with proteoglycans, leading to the accumulation of CPPs at the cell surface. The electrostatic interaction has been reported
to trigger an energy-dependent endocytotic process [108]. Micropinocytosis and clathrin- and caveolin–dependent endocytosis have also been reported to mediate internalisation of CPP’s and these mechanisms may occur simultaneously [105]. The ability of a peptide to escape from an endosome is therefore important and can be a rate-limiting step in CPP-mediated drug delivery. Several other factors may also affect the cellular uptake including the secondary structure of a CPP, the nature, type and concentration of its cargo and the membrane composition of the target cell [109]. For example, secondary amphipathic CPPs such as Penetratin have weak affinity for zwitterionic membranes but can accumulate at high concentrations on anionic membranes [109]. Cellular uptake of CPPs has also been found to occur independently of endocytosis. This is favoured when the concentration at the cell surface is increased and leads to a more cytoplasmic distribution of CPPs [110]. Several mechanisms for this translocation have been proposed but direct translocation data are scarce, and it remains challenging to predict due to the complex of factors at play.

The in vivo potency of CPPs was first demonstrated in 1999 when a tat-β-galactosidase fusion protein showed that delivery into almost all tissues was possible following intraperitoneal injection in mice [111]. Since then, CPP-based delivery systems have been used to deliver peptides and proteins into cells targeting various processes such as stimulating cytotoxic immunity and targeting diseases including asthma, ischemia, diabetes and cancer. A principal application has been in anti-proliferative treatments for cancer, with much success reported. Many of these studies have used Tat or Penetratin conjugated to various peptides including those derived from tumour suppressor p53, proapoptotic peptides and inhibitors of cyclin-dependent kinases involved in cell cycle progression [112-115]. Successful translation to the clinic for these treatments has been
slow. First generation CPPs often had issues with cell, tissue or organ specificity. Research into cell, organ and disease selective strategies is therefore underway. As entry to the cell is often mediated through endocytic pathways, endosomal escape is also critical for the cargo to exert its effects in the cell. It has been confirmed by fluorescence-based methods and through mass spectrometry that in many cases, CPP-cargo conjugates remain trapped in the endocytic pathway \[116, 117\]. Cargo that remains trapped within endosomes cannot reach their cytosolic or nuclear targets and will not display any biological activity. Furthermore, they may be subject to degradation in late endosomes or lysosomes through hydrolases or acidic pH \[118\].

2.5.2 Phylomer-1746
A new class of CPPs has been recently developed by Phylogica Ltd. (a Perth based biotechnology company) through the screening of their Phylomer libraries \[119\]. The phylomer libraries contain genome fragments derived from biodiverse bacteria and archaea species. When compared with random peptide libraries, sequences derived from natural peptides provide more secondary and tertiary configurations that have been optimised for biological activity and interaction, making them a useful reservoir for many applications \[120\]. Several CPP phylomers were selected for potent internalisation through the plasma and nuclear membranes. A Split–complementation Endosomal Escape (SEE) assay was developed and used to visualise cytosolic internalisation of CPPs, differentiating internalisation from endosomal entrapment \[121\]. One phylomer which was identified as showing superior internalisation and endosomal escape is a 38 amino acid sequence, Phylomer-1746 (unpublished). A recombinant fusion protein comprising of Phylomer-1746 linked to the Omomyc sequence (1746-Omomyc) was produced in bacteria and has demonstrated substantial activity in murine and human
basal-like cell lines (unpublished). It is of interest to investigate the mechanisms involved and address Myc inhibition in cancer cell lines treated with this protein.

2.6 Recombinant versus Synthetic Production of Therapeutic proteins

Omomyc and 1746-Omomyc are large molecules, with Omomyc alone comprising 92 amino acids. The size of a therapeutic protein is important for several reasons. Larger molecules usually have lower tissue penetration and may have a lower level of activity per unit mass [122]. The key benefit to reducing the size of a therapeutic protein, however, is an increase in the technological options available for its manufacture. Generally, peptides up to 100 amino acids in length can be produced chemically through solid phase peptide synthesis (SPPS). As the length of a peptide increases, the yield of pure peptide decrease due to poor coupling efficiencies, making this technology unsuitable for larger proteins [123]. There are several potential benefits of utilising SPPS compared with recombinant expression. Recent developments have allowed large-scale chemical synthesis of small and medium sized peptides to become a viable option with reduced manufacturing costs [124]. Recombinant production can be expensive as it involves complex manufacturing processes and the development of stable cell lines. Chemical synthesis also allows for greater chemical diversity. For example, unnatural and D-amino acid substitutions can be made and may provide benefits such as greater stability in plasma [125]. There can also be benefits in terms of product purity. The quality and purity of recombinant molecules are not always optimal. Chemical synthesis allows the product to be easily separated from impurities and side products and also provides a reduced risk of contamination with other biologics such as viruses [123].
Although Omomyc is large, the amino acid substitutions allowing for its interaction with Myc all occur within a small 15 amino acid region. It is therefore of interest to determine if a truncated version of Omomyc (Omi), a 28 amino acid peptide derived from the interference region of Omomyc, would also show sufficient activity in inhibiting Myc when conjugated to a CPP.
3. Hypotheses and Aims

Hypotheses:

That interference peptides 1746-Omomyc, 1746-Omi and Penetratin-Omi will inhibit Myc in TNBC cell lines leading to reduced proliferation and/or cancer cell death.

Aims:

1. To investigate the level of Myc expression in a panel of tumorigenic and non-tumorigenic human and murine mammary cell lines using qRT-PCR. The murine panel includes one non-tumorigenic (NIH-3T3), and three triple-negative cell lines (T11, A1.8, B.15). The human panel includes one non-tumorigenic (HDEF), three triple negative (SUM149, SUM159, MDA-MB-231) and two hormone receptor positive (MCF7 and ZR-75-1) breast cancer cell lines.

2. To determine the IC$_{50}$ of interference peptide 1746-Omomyc in the same panel of cell lines and investigate whether the response is Myc or tumour subtype dependent.

3. To determine the half maximal inhibitory concentration (IC$_{50}$) of the truncated peptide (Omi) conjugated to both a traditional CPP, Penetratin (Penetratin-Omi) and phylomer-1746 (1746-Omi) in T11 cells.

4. To investigate the mechanisms of inhibition of the three peptides using a Ki-67 Proliferation Assay and an Annexin V and Propidium Iodide staining kit.

5. To investigate Myc inhibition by evaluating the expression of known downstream Myc targets using qRT-PCR.
4. Materials and Methods

4.1 Cell lines

The NIH-3T3, MCF7, ZR-75-1, HDEF and MDA-MB-231 cell lines were acquired from ATCC. SUM159 and SUM149 cell lines were obtained from Asterand Bioscience. The T11, A1.8 and B.15 lines were gifted from collaborators. T11, A1.8 and B.15 cells were grown in RPMI-1640 media with 10% Foetal Bovine Serum (FBS). NIH-3T3, HDEF and MDA-MB-231 cells were grown in DMEM with 10% FBS. SUM159 cells were grown in Hams F-12 media with 5% FBS, 1 µg/ml of hydrocortisone and 1.25 µg/ml insulin. SUM149 cells were grown in Hams F-12 media with 10% FBS. MCF7 cells were grown in MEM with 10% FBS and 1% each of sodium pyruvate, sodium bicarbonate and non-essential amino acids. ZR751 were grown in RPMI-1640 with 10% FBS, 2 mM glutamine and 1mM Sodium Pyruvate. All media had 1% Antibiotic-Antimycotic added.

4.2 Peptides

1746-Omi, Penetratin-Omi, Omi, Penetratin, FITC-1746 and the Mutant Penetratin-Omi were synthesised by China Peptides Co., Ltd, Shanghai, China using SPPS. The peptides came in the form of a lyophilized powder. Each peptide came with a High-Performance Liquid Chromatography report indicating the product purity and a Mass Spectral Analysis report. All peptides had a high purity grade of above 95% making them suitable for quantitative as well as qualitative studies. Peptides were stored at -20°C. At first use peptides were diluted in phosphate-buffered saline (PBS) and frozen at -20°C in small aliquots for use in experiments. 1746-Omomyc, Omomyc and 1746 were supplied by Phylogica Ltd. who produced them recombinantly using an expression vector in bacteria.
4.3 Quantifying Myc mRNA expression in mouse and human cell line panels

4.3.1 RNA extraction and quantification

All cell lines wells were grown on 10cm tissue culture plates until they were approximately 90% confluent in the appropriate media. Cells were washed with PBS before trypsin was added to detatch cells from the plate. Trypsin was inactivated by adding media and cells were centrifuged to produce a pellet. Pellets were washed twice with PBS. Cells were resuspended in 600µL of Trizol and frozen at -80°C until needed. All samples were defrosted in their tubes on ice. 120µL of chloroform was added and tubes were vortexed at high speed for 15 seconds. Samples were incubated on ice for 3 minutes and centrifuged (4°C, 12,000 rpm, 20 minutes) to separate. The supernatant containing the RNA was transferred to a new Eppendorf tube. 300µL of cold (-20°C) isopropanol was added and samples were vortexed lightly. Samples were incubated on ice for 10 minutes and then stored at -20°C for 1 hour. Samples were then centrifuged (4°C, 13,000 rpm, 40 minutes). Supernatant was discarded and pellets were washed twice with 75% ethanol by adding ethanol to tubes, centrifuging for 10 minutes at 4°C and discarding the ethanol. Pellets were air dried at room temperature before being resuspended in 40µL nuclease free water and incubated at 4°C for 1 hour. The concentration of RNA in each sample was quantified using a NanoDrop spectrophotometer which calculates the concentration based on the light absorbance at 260 nm. All samples showed 260nm/280nm absorbance ratios above 1.8 and 260nm/230nm absorbance ratios above 2.2 indicating the samples were free of protein, phenols or other contaminants. Samples were then frozen at -20°C until required.
4.3.2 cDNA synthesis and qRT-PCR

The RNA samples were converted to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with the amount of RNA added calculated to give each sample an equal final RNA concentration. cDNA was synthesised using the BioRad S1000 Thermal Cycler. Samples were stored at -20°C until needed. qRT-PCR was performed using TaqMan Fast Universal Master Mix and the ViiA 7 Real-Time PCR system (Applied Biosystems). Each sample was run in triplicate with both human and mouse Myc and GAPDH Taqman® probes. Non-template controls were included for each probe.

4.3.3 Analysis of data using the Delta Delta CT method

The amplification plots were checked to ensure that triplicates for each sample had minimal variation. The amplification plots of the controls were checked and there was no evidence of contamination. A Ct value was obtained for each sample and the analysis was calculated manually using Excel. The mean Ct value and standard error for each sample were calculated from the triplicate data. The error was calculated from the standard error values for Myc and GAPDH for each cell line. The Delta Ct Standard Errors were calculated using the NIH-3T3 cell line as the control for the mouse panel and the HDEF line as the control for the human panel. Delta Ct was calculated for GAPDH and Myc by subtracting the mean sample Ct value from the mean control (NIH-3T3 or HDEF) Ct value. Double Delta Ct was calculated by subtracting the Delta Ct from the housekeeping gene (GAPDH) from the Delta Ct of our gene of interest (Myc). The ratio of expression was calculated by exponentiation of the delta delta Ct.
4.4 Cell Viability Assays

4.4.1 Peptide Treatment and CellTiter-Glo 2.0® Assay

In each experiment, cells were plated in 96 well plates at 3000 cells per well and incubated in 5% CO₂ at 37°C for 24 hours before treatment. The calculated amount of peptide for each concentration was added to media in tubes and lightly vortexed. Media was then aspirated from the wells and 100µL of the media-peptide mix added to each well. Triplicate wells of cells were treated with each concentration of peptide tested. A control well with no cells was also treated for each concentration to act as a background for the luminescence when the assay was read. Plates were read after 24 hours incubation using a CellTiter-Glo® 2.0 Assay from Promega. The reagent was added, plates shaken for 2 minutes then the luminescence measured using the Wallac Envision™ 2102 Multilabel Reader after 10 minutes. This assay indicates the number of viable cells by quantitating the amount of ATP present. Each experiment was repeated three times, although in some cases only two repeats were carried out due to limitations on time and resources.

4.4.2 Calculating IC₅₀ values

Readouts from the CellTiter-Glo 2.0 Assay were first processed using excel to get an average value for each well over the eight iterations read and then the average value for each concentration tested. The background value recorded for the treatment wells containing no cells was then subtracted. The luminescence value for the control wells with no peptides added was taken to indicate 100% viability and the rest of the data compared to this value to give the percentage of viable cells under each concentration of peptide tested. The graphs and IC₅₀ values were produced using GraphPad Prism 7. The concentrations were transformed using a Log conversion and a sigmoidal standard curve fitted to the data.
4.4.3 Statistical Analysis

All p-values comparing treatments were based on independent sample t-tests calculated using SPSS. The relationship between Myc level and response to 1746-Omomyc were analysed using linear regression in SPSS. A Log transformation of the response to 1746-Omomyc was used for the human panel. Q-Q plots of observed versus expected values indicated the assumption of normally distributed data was upheld. The assumption of homeoscadacity was checked by looking at residuals versus predicted values and did include some deviations.

4.5 Ki-67 Proliferation Assays

4.5.1 Treatment procedure and conditions for Ki-67 Assay

Cells were seeded at 3,000 cells per well in transparent 96 well plates and incubated in 5% CO$_2$ at 37°C for 24 hours before being treated. A second white bottomed control plate was also seeded with cells. Four wells of cells were treated with each peptide being tested at 2.5µM for 1746-Omomyc, 25µM for Penetratin-Omi and the mutant Penetratin-Omi and 9µM for the positive control (SV40-EN1 i pep) as well as four control wells with no treatment. Triplicate wells on the control plate were also subjected to each treatment.

Cells were incubated for 24 hours. Cell viability was tested on the control plate using a CellTiter-Glo 2.0 Assay to ensure all peptides were active and the test plate was fixed and stained.

4.5.2 Immunofluorescence staining

Media was aspirated and the cells fixed with 4% Formaldehyde in PBS. Cells were washed in PBS and permeabilised with 0.5% Triton™ X-100 in PBS for 15 minutes at 4°C. Cells were washed with PBS and blocked for 1 hour at room temperature with 4%
Bovine Serum Albumin (BSA) in PBS. Blocking solution was aspirated and the cells treated with a 1:400 dilution of a Ki-67 Mouse monoclonal antibody (mAb) purchased from Cell Signalling Technology® diluted in 1% BSA in PBS. The fourth well for each treatment had only 1% BSA in PBS added with no primary Ki-67 antibody to act as a control. Cells were incubated on a shaker at 4°C overnight. Cells were washed three times with 0.1% Tween 20 in 1% BSA in PBS and a Goat anti-Mouse IgG secondary antibody, Alexa Flour® 488 conjugate was added in a 1:1000 dilution. The plate was wrapped in foil and incubated at room temperature on a shaker for 1 hour. Cells were washed three times with washing solution and then stained with a Hoechst nuclear stain. Cells were washed three times in washing solution and two times in PBS and were stored at 4°C covered in PBS and wrapped in foil until reading.

4.5.3 Imaging and Data Analysis

Images were captured using the Olympus IX71 Inverted Fluorescence Microscope and AnalySIS image capture software. Images used for quantification were at 10x magnification with the exposure set manually and kept constant across all images. Images of three fields of view were taken for each well. The total number of cells for each field of view was counted manually from the image with the Hoechst filter using the ImageJ cell counter plugin. The number of Ki-67 positive cells was determined by counting the number of fluorescent cells obviously visible in the corresponding image using the green filter image with the brightness and contrast kept constant for all images counted. Statistics and graphs were produced using GraphPad Prism 7. The mean and standard deviation were calculated using the percentage of Ki-67 positive cells in each field of view.
4.6 Annexin V and Propidium Iodide Assay

4.6.1 Peptide treatments and procedure for Annexin V and PI Assay

T11 cells were plated in 6 well plates at ~90000 cells per well (scaled up from the 3,000 cells per well used in previous experiments on 96 well plates) and incubated for 24 hours before being treated. One well was treated with each of the following: 1746-Omomyc, Omomyc and 1746 at 2.5µM, Penetratin-Omi, Mut Penetratin-Omi and Penetratin at 25µM and a control well with no peptide added. Triplicate wells of T11 cells in a separate 96 well plate were also treated at the same time with the same preparation of peptides to act as a control on the level of cell viability under each treatment. After 24 hours incubation, cell viability was measured in the control plate using a CellTiter-Glo 2.0 Assay and an Invitrogen™ Dead Cell/ Apoptosis Kit was used to quantify the number of living, dead and early apoptotic cells in the treated cells in the 6 well plates. The supernatant was collected for each sample. Adherent cells were removed using trypsin, added to the supernatant and centrifuged. Supernatant was discarded and cells washed in cold PBS. Each sample of cells was resuspended in 100µL Annexin binding buffer. 5 µL of the FITC-Annexin V and 1µL of a 100µg/ml Propidium Iodide solution were added to each sample and incubated at room temperature for 15 minutes. A further 400µL of Annexin binding buffer was added and samples kept on ice until reading. Cells were counted using the BD FACS Aria II Special Order System, USA. FITC was detected with the blue laser at 488nm and Propidium Iodide with the Yellow/Green laser at 552nm with acquisition criteria of 100,000 events per tube. Single cells were gated into four populations: Annexin V+ PI+, Annexin V−, PI−, Annexin V+ PI− and Annexin V− PI+. 
4.7 mRNA expression of downstream Myc targets

4.7.1 Treatment procedure and conditions

T11 cells were seeded onto a 6 well plate at ~90000 cells per well and incubated at 37°C for 24 hours. One well of each plate was treated with 1746-Omomyc, 1746 and Omomyr at 5 µM, Penetratin-Omi and Mut Penetratin-Omi at 50µM and a control well with no peptide added. The cells were collected after 3 hours by aspirating media and resuspending in 400µL trisol. Samples were frozen at -80°C. RNA was extracted and quantified using the same method as above for the Myc mRNA quantifications.

4.7.2 qRT-PCR and data analysis

cDNA was synthesised and qRT-PCR carried out as described in 4.3.2. Triplicate wells were run for each of the samples with each of the probes: MYC, CCND1, MINA, E2F2, CDKN1A and GAPDH (Taqman®). Non-template control wells for each probe were included. The data was analysed using the Delta Delta Ct method as described in 4.3.3 using the non-treated cells as the control and GAPDH as the reference gene.
5. Results

5.1 Myc mRNA expression in cell line Panels

5.1.1 Description of cell lines

To identify the most suitable cell lines to use in experiments aimed at inhibiting Myc and determine if Myc levels are predictive of a response to the 1746-Omomyc peptide, Myc mRNA levels were quantified in a panel of murine (NIH-3T3, T11, A1.8, B.15) and human (HDEF, SUM149, SUM159, ZR-75-1, MCF7, MDA-MB-231) cell lines using qRT-PCR.

The NIH-3T3 cell line is a non-tumorigenic line of immortalised mouse embryonic fibroblasts and was used as a non-tumorigenic reference/control in the mouse panel. The other three murine cell lines tested are tumorigenic. The T11 cell line is a p53 null, mouse mammary tumour model [126, 127]. It is characteristic of the claudin-low subtype and is negative for ER, PR and HER-2 overexpression. Both the A1.8 and B.15 cell lines were generated from BRCA1 deficient mouse mammary tumours [128]. They are both of the basal subtype and also display a triple-negative phenotype.

HDEF cells are a non-tumorigenic cell line derived from normal adult human dermal fibroblasts and were used as a reference/control to quantify Myc levels in the human panel. SUM149 and SUM159 are both p53 deficient tumorigenic human cell lines of the triple-negative phenotype. SUM149 cells have been characterised as basal-like whilst SUM159 cells are characteristic of the claudin-low subtype displaying high enrichment for epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features [19]. MDA-MB-231 is also a tumorigenic human cell line with a triple-negative phenotype [129]. These cells are typically characterised as being of the
basal subtype, however, they have also been shown to display characteristics consistent with claudin-low tumours [19]. ZR-75-1 and MCF7 are both hormone receptor positive tumorigenic human breast cancer cell lines of the luminal subtype. The characteristics of the cell lines used are summarised in Table 3.

Table 3: Cell lines tested and their characteristics.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>BC Subtype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-3T3</td>
<td>Mouse</td>
<td>N/A</td>
<td>Murine Embryonic Fibroblasts</td>
</tr>
<tr>
<td>T11</td>
<td>Mouse</td>
<td>Claudin-low</td>
<td>P53mut, TN</td>
</tr>
<tr>
<td>A1.8</td>
<td>Mouse</td>
<td>Basal-like</td>
<td>BRCA1+, TN</td>
</tr>
<tr>
<td>B.15</td>
<td>Mouse</td>
<td>Basal-like</td>
<td>BRCA1+, TN</td>
</tr>
<tr>
<td>HDEF</td>
<td>Human</td>
<td>N/A</td>
<td>Human Dermal Fibroblasts</td>
</tr>
<tr>
<td>SUM159</td>
<td>Human</td>
<td>Claudin-low</td>
<td>P53mut, TN</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human</td>
<td>Claudin-low</td>
<td>P53mut, TN</td>
</tr>
<tr>
<td>SUM149</td>
<td>Human</td>
<td>Basal-like</td>
<td>P53mut, TN</td>
</tr>
<tr>
<td>MCF7</td>
<td>Human</td>
<td>Luminal</td>
<td>ER+, PR+</td>
</tr>
<tr>
<td>ZR751</td>
<td>Human</td>
<td>Luminal</td>
<td>ER+</td>
</tr>
</tbody>
</table>

5.1.2 Highest *Myc* expression in the triple-negative breast cancer claudin-low cell lines

In both the murine and human panel of cell lines, the highest *Myc* expression was seen in the claudin-low subtype. In the murine panel, *Myc* mRNA levels were elevated in all three of the tumorigenic cell lines tested when compared to the non-tumorigenic NIH-3T3 cells (Figure 3A). The T11 cells (claudin–low) had the highest level with a 1.35 fold expression of *Myc* compared with the control. The two basal-like cell lines showed similar expression levels with a 1.28 fold increase for A1.8 and a 1.22 fold increase for B.15 cells.

*Myc* mRNA levels in the human panel were normalised to levels in the non-tumorigenic HDEF cells. All cell lines tested showed elevated *Myc* expression in comparison to HDEF.
The luminal ZR-75-1 cells showed the smallest increase of 1.33 fold. The luminal MCF7 cells and basal-like SUM149 cells expressed similar levels of *Myc* at 4.96 and 4.71 fold increased expression. The highest *Myc* expression was seen in the claudin-low subtype with a 5.44 fold increase in MDA-MB-231 cells and a 22.34 fold increase in SUM159 cells.

![Figure 3: Myc mRNA levels are highest in the claudin-low cell lines. A. c-Myc mRNA levels determined by qRT-PCR in a murine panel of cell lines. Calculated using the Delta Delta Ct method with GAPDH as the reference gene and normalised to expression levels in NIH-3T3 cells. B. c-Myc mRNA levels determined by qRT-PCR in a human panel of cell lines. Calculated using the Delta Delta Ct method with GAPDH as the reference gene and normalised to HDEF c-Myc expression levels. Error bars represent Standard Error of the Mean (SEM) from a single experiment.](image)

**5.2. 1746-Omomyc Dose Responses in panels of cell lines**

**5.2.1 Greatest response to 1746-Omomyc in triple-negative claudin-low cell lines**

The 1746-Omomyc peptide was delivered in a range of doses to both the murine and human panels of cell lines. The percentage of viable cells after 24 hours treatment was quantified against untreated controls using a CellTiter-Glo cell viability Assay. In the murine panel, the largest decrease in the number of viable cells was seen in the claudin-low T11 cells with an IC₅₀ value of 2.123µM (95% CI 1.833 to 2.46) (Figure 4A). The basal-like B.15 were also responsive to the peptide with an IC₅₀ of 2.298µM (95% CI 1.998 to 2.644). A1.8 cells were affected to a lesser extent at lower concentrations but substantially at the highest concentration. NIH-3T3 cells were far less responsive. At the
maximum concentration tested of 15µM, the percentage of viable NIH-3T3 cells was reduced to a mean of 42.43% of that seen in non-treated controls. In all three tumorigenic cell lines tested the mean percentage of viable cells at this concentration was much smaller at 2.15% in T11, 6.85% in B.15 and 5.44% in A1.18 cells.

In the human panel, the greatest response to the 1746-Omomyc peptide was also seen in a claudin-low cell line, SUM159, which had an IC₅₀ of 2.177µM (95% CI 1.85 to 2.563). (Figure 4B). The basal-like SUM149 were also substantially affected with an IC₅₀ of 3.417µM (95% CI 2.847 to 4.1). In contrast, the non-tumorigenic HDEF cells were not affected as greatly by the peptide with the number of viable cells measured at 84.71 percent of that seen in the non-treated control cells at the maximum concentration tested of 15 µM. The results were similar for the luminal MCF7 cells with cell viability measured at a mean of 86.84 percent seen in non-treated controls still viable after 24 hours treatment at 15µM. Luminal ZR-75-1 cells were not greatly affected with peptide concentrations up to 5µM, but cell viability dropped to 41.66 percent of the control at a concentration of 15µM. Results were similar in the claudin-low MDA-MB-231 cells which showed no response at 2.5µM but was inhibited at concentrations of 5µM and 15µM.

Dose responses of the 1746 and Omomyc peptides were also tested at a range of concentrations up to 15µM in a representative cell line to act as controls. The T11 cell line was chosen as it showed the greatest reduction in cell viability when treated with the 1746-Omomyc peptide. Neither the 1746 or Omomyc peptides produced a decrease in cell viability when administered alone (Figure 4C).
Response to 1746-Omomyc correlates with Myc RNA levels in Human Panel

In both the murine and human panel, the response to 1746-Omomyc was greatest in the cell line with the greatest Myc mRNA expression. Linear regression analysis was undertaken to determine if there was a significant relationship between Myc RNA levels and the response to the 1746-Omomyc peptide. Data from the percentage of viable cells at 2.5µM was used as this was the closest data point to the IC$_{50}$ of the most responsive cell lines in both the murine and human panels. In the murine panel, although the cell line with the highest Myc levels (T11) had the greatest response, a significant relationship

Figure 4: Dose response curves of 1746-Omomyc on (A) murine cell line panel and (B) human cell line panel. Tested at concentrations ranging from 0.25 to 15µM. Cells were treated for 24 hours and cell viability assessed using CellTiter-Glo Assays. The percentages of viable cells were normalised to non-treated control cells. Data points are the mean of three independent experiments for NIH-3T3, T11, SUM159 cell lines and two experiments for the remaining cell lines. Error bars represent SEM. C. Dose response of 1746-Omomyc with 1746 and Omomyc as controls presented as mean and SEM of three independent experiments. Where error bars can’t be seen they are smaller than the symbols.
between response to 1746-Omomyc and Myc level was not found (p = 0.309). This result, however, was based on a very small dataset with Myc levels being in a very small range, limiting the value of such an analysis. In the human panel, a significant linear relationship between Myc RNA level and response to the 1746-Omomyc was found with a lower percentage of viable cells compared to the control after treatment in cells with higher Myc levels (p = 0.033).

5.3 1746-Omi and Penetratin-Omi Dose Responses

A truncated 28 amino acid version of the Omomyc peptide was synthesised (Omi) which corresponds to amino acids 54 to 81 of the Omomyc peptide (See Table 2). Rather than containing the entire basic helix-loop-helix-leucine zipper region of Omomyc, this peptide corresponds only to the critical portion of the protein that is involved in dimerization [6]. The Omi peptide linked to the CPPs 1746 (1746-Omi) and Penetratin (Penetratin-Omi) were produced via SPPS.

5.3.1 CPP-Omi conjugates have increased IC₅₀ values

Dose-response data were obtained for 1746-Omi, Penetratin-Omi and controls (1746, Penetratin, and Omi) in T11 cells treated for 24 hours using a CellTiter-Glo cell viability Assay. The IC₅₀ for 1746-Omi was 26.24µM (95% CI 22.38 – 30.76), which was nearly 10 fold higher than the concentration needed for 50% cell death using the 1746-Omomyc peptide (Table 4). Penetratin-Omi produced an IC₅₀ of 20.6 µM (95% CI 16.19 – 26.2). When administered alone neither CPP nor Omi caused a reduction in the number of viable cells (Figure 5).
5.3.2 Greater reduction in cell viability produced by Penetratin-Omi than 1746-Omi

Penetratin-Omi was found to reduce the number of viable T11 cells significantly more than the 1746-Omi peptide at concentrations of 10, 15 and 20 µM from a mean of 94.4% for 1746-Omi to 83.3% for Penetratin-Omi at 10 µM (p = 0.035), from 87.0% to 67.1% at 15 µM (p = 0.026) and from 80.9% to 52.1% at 20 µM (p = 0.012) (Figure 5).

To determine its suitability for internalisation studies, cell viability of T11 cells treated with a FITC labelled 1746 peptide was also measured. The number of viable cells was reduced greatly to 59.8 ± 4.14% (mean ± SEM) of the control at the maximum concentration tested of 50 µM. In comparison, cell viability was 98.8 ± 0.16% (mean ± SEM) of the control for unlabelled 1746 at the same concentration (Sup. Figure 1).

Figure 5: Greater reduction in cell viability for Penetratin-Omi than 1746-Omi. Dose response data of Penetratin-Omi, 1746-Omi, Penetratin, 1746 and Omi in T11 cells treated at concentrations from 0.25 to 50 µM for 24 hours and cell viability assessed using CellTiter-Glo Assays. Percentage of viable cells was normalised to non-treated control cells. All data points are presented as mean ± SEM for three independent experiments.

### Table 4: Increased IC$_{50}$ in CPP-Omi conjugates
Comparison of peptides IC$_{50}$ in T11 cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$ in T11 cells (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1746-Omomyc</td>
<td>2.12</td>
</tr>
<tr>
<td>1746-Omi</td>
<td>26.24</td>
</tr>
<tr>
<td>Penetratin-Omi</td>
<td>20.60</td>
</tr>
<tr>
<td>Mutant Penetratin-Omi</td>
<td>24.95</td>
</tr>
</tbody>
</table>
5.3.3 CPP-Omi conjugates show selectivity towards cancer cell line

1746-Omi and Penetratin-Omi were also tested in the non-tumorigenic NIH-3T3 cell line. In this cell line neither peptide produced any decrease in cell viability even at the highest concentration tested of 50µM (Figure 6A, 6B).

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 6: Penetratin-Omi and 1746-Omi show selectivity towards the cancer cell line.** A. Dose-response data of 1746-Omi in T11 and NIH-3T3. B. Dose-response data of Penetratin-Omi in T11 and NIH-3T3. Treated at concentrations from 0.25 to 50µM for 24 hours and cell viability assessed via CellTiter-Glo Assays. Percentage of viable cells was normalised to non-treated control cells. All data points are presented as mean ± SEM for three independent experiments. Where no error bars can be seen they are smaller than the symbols.

5.3.4 Mutant Penetratin-Omi also reduces cell viability

Given the greater potency and favourably smaller size of Penetratin-Omi over 1746-Omi and the greater characterisation of the penetratin CPP, Penetratin-Omi was chosen for use in further experiments. An altered version of the Penetratin-Omi peptide was produced via SPPS to act as a control in which the four amino acids critical in dimerization were replaced by alanine (Mutant Penetratin-Omi). The altered version of the peptide was also found to produce a decrease in cell viability with an IC$_{50}$ of 24.95µM (95% CI 23.06 – 26.99) (Figure 7). The difference in the percentage of viable cells when treated with Penetratin-Omi and Mutant Penetratin-Omi was found to be significantly different at concentrations of 10, 15 and 20µM (p = 0.025, 0.045 and 0.047 respectively). At 10µM,
the mean percentage of viable cells when compared to an untreated control was 83.33% for Penetratin-Omi and 93.08% for the Mutant. At 15µM, viability was 67.06% for Penetratin-Omi and 83.39% for the Mutant. At 20µM, Penetratin-Omi reduced the number of viable cells to 52.07% whilst the Mutant reduced the number to 67.29%.

Figure 7: Mutant Penetratin-Omi also reduces cell viability. Dose-response data of Penetratin-Omi and Mutant Penetratin-Omi in T11 cells. Treated at concentrations from 0.25 to 50µM for 24 hours. Cell viability assessed via CellTiter-Glo Assays. Percentage of viable cells was normalised to non-treated control cells. All data points are presented as mean ± SEM for three independent experiments. Where no error bars can be seen they are smaller than the symbols.

5.4. Proliferation and Dead Cell/Apoptosis Assays

The 1746-Omomyc, 1746-Omi, Penetratin-Omi and Mutant Penetratin-Omi peptides all produced a reduction in viable cancer cells. The CellTiter-Glo Assay used to measure this reduction is based on quantitating the amount of ATP present in a sample, indicating the presence of metabolically active cells. This does not inform us of the extent to which the reduction in the number of viable cells was produced via reduced proliferation or through cell death. To get a clearer idea of the extent to which the reduced cell viability was due to a reduction in proliferation or through cell death a Ki-67 Proliferation Assay and an Annexin V and Propidium Iodide Dead Cell/Apoptosis Assay were performed.

5.4.1 1746-Omomyc reduces viable cells through reduced proliferation in T11 cells

Ki-67 is a useful marker of proliferation as it is universally expressed in proliferating cells and is detectable in G1, S, G2 and mitosis, but is absent in quiescent cells in the G0 resting
phase [130]. Ki-67 positive cells were quantitated in peptide treated T11 cells using a Ki-67 antibody and fluorescently labelled secondary antibody. In the control, 89.36 ± 0.657% (mean ± SEM) of cells were positive for the Ki-67 protein indicating that they were proliferative (Figure 8). This proportion was significantly reduced (p < 0.001) in the cells treated with the 1746-Omomyc peptide to 46.87 ± 1.422% (mean ± SEM). In the cells treated with the Penetratin-Omi, 76.21 ± 1.414% (mean ± SEM) were still proliferative. This was significantly less than seen in the control cells (p < 0.001) but significantly more than in cells treated with 1746-Omomyc (p < 0.001). The proportion of proliferative cells when treated with Mutant Penetratin-Omi, was not significantly different from what was seen in the cells treated with Penetratin-Omi (p = 0.342) with 78.43 ± 1.785% (mean ± SEM) positive for Ki-67.

**Figure 8: 1746-Omomyc reduces proliferation in T11 cells.** T11 cells treated for 24 hours at 2.5µM for 1746-Omomyc and 25µM for Penetratin–Omi and Mutant Penetratin-Omi. Data is mean number of Ki-67 positive cells over nine fields of view at 10x magnification for each treatment. Error bars are standard deviation (*** represents p < 0.0001).

### 5.4.2 Penetratin-Omi and Mutant reduce cell viability through cell death in T11 cells
Annexin V is able to identify early apoptotic cells by binding to phosphatidylserine which is translocated from the inner to the outer leaflet of the plasma membrane during the process of apoptosis before the cell loses membrane integrity [131]. Propidium Iodide is
impermeant to live and early apoptotic cells, but stains dead or late apoptotic cells with red fluorescence.

T11 cells were stained using a FITC-Annexin V and Propidium Iodide staining kit after being treated with the peptides and controls for 24 hours at the same concentrations as used in the Ki-67 assay. Cells were counted using FACS (Figure 9). In the control, the majority of cells (94.7%) were negative for both Annexin V and PI, indicating non-apoptotic, viable cells. Only 0.2% of cells were Annexin V⁺, PI indicator cells in early apoptosis. 1.9% of the control cells stained Annexin V⁺, PI⁺ corresponding to dead cells, with the remaining 3.2% of cells Annexin V⁺, PI⁺. These cells may be in late apoptosis or dead. This assay is unable to distinguish between dead cells that have undergone apoptotic death versus a necrotic pathway as once membrane integrity is lost necrotic cells may also stain positive for Annexin V. All control peptides (Omomyc, 1746 and Penetratin) produced results very similar to the untreated control cells (Sup. Figure 2).

After treatment with 1746-Omomyc, the majority of cells were also viable and non-apoptotic (85.9%). Early apoptotic cells were at the same level seen in the control at 0.2% with the remaining 13.9% of cells PI⁺. The Penetratin-Omi treated cells had a much lower proportion of live cells at 37.0%, a higher proportion of early apoptotic cells at 1.3% and a large number of PI⁺ cells at 61.7%. The mutant Penetratin-Omi treated cells had a higher proportion of live cells at 77.7%, 2.1% early apoptotic cells and a total of 20.2% PI⁺ cells.

The proportion of dead cells after treatment with each peptide are not directly comparable as the peptides have different IC₅₀ values. A separate plate of cells was treated at the same time, with the same preparations of peptides and a CellTiter-Glo cell viability Assay was
used to determine the number of viable cells after each treatment when compared to a control. The data from the two assays is compared in Table 5. The number of viable cells compared to a control after 24 hours incubation with 1746-Omomyc at 2.5µM was reduced to 38.7%. The proportion of live cells in the corresponding sample assessed via FACS, however, was not greatly reduced with 86.1% of cells still viable (only 8.8% lower than the control) with the number of dead cells being very low. This supports the result of the Ki-67 proliferation assay which showed a marked reduction in the number of proliferating cells following treatment with this peptide. When incubated for 24 hours with Penetratin-Omi at 25 µM, the number of viable cells was reduced to 34% of the control. Most of this reduction is accounted for by dead cells with the total number of PI-positive cells counted at 61.7%. This result is also supported by the result of the Ki-67 proliferation assay which showed only a small reduction in proliferating cells compared to the control. In the cells treated with Mutant Penetratin-Omi for 24 hours at 25µM, the number of viable cells was reduced to 48.9% of the control based on the CTG assay. The
proportion of PI-positive cells was lower than expected at 20.2%, however, there was no corresponding decrease in the number of proliferating cells as was seen for the 1746-Omomyc peptide.

Table 5: Comparison of the percentage of viable cells after treatment compared to a control versus percentage of viable cells after treatment as a percentage of the sample.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC_{50} of peptide in T11</th>
<th>Viable cells (% of Control) CTG Assay</th>
<th>Total Viable cells (%) Annexin V/PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
<td>94.7</td>
</tr>
<tr>
<td>1746-Omomyc (2.5 µM)</td>
<td>2.12</td>
<td>38.7</td>
<td>85.9</td>
</tr>
<tr>
<td>Penetratin-Omi (25 µM)</td>
<td>20.60</td>
<td>34.0</td>
<td>37.0</td>
</tr>
<tr>
<td>Mutant Penetratin-Omi (25 µM)</td>
<td>24.95</td>
<td>48.9</td>
<td>77.7</td>
</tr>
</tbody>
</table>

*Total viable cells: Results of FACS analysis on FITC-Annexin V and PI stained T11 cells treated for 24 hours. Viable cells (% of control) based on CellTiter-Glo 2.0 Viability Assay with same treatments on separate plate*

5.5 qRT-PCR on Downstream Myc targets

5.5.1 Selection of downstream Myc targets

To determine if the mechanism of action of the Penetratin-Omi and Mutant Penetratin-Omi peptides was consistent with Myc inhibition, their effect on known downstream Myc targets was investigated. An RNA-Seq analysis of T11 cells treated with 1746-Omomyc and controls had previously been carried out (data are still being analysed by collaborators). Five genes known to be responsive to Myc protein levels were selected that were found to be either up- or down-regulated in the RNA-Seq data.

The gene for Myc-induced nuclear antigen (MINA) was chosen as it is a direct target gene of Myc, interacting through an E-box site in the genes promoter. MINA mRNA and protein levels are reduced in response to a reduction in Myc protein levels [132]. MINA has been shown to play an important role in cellular proliferation and is overexpressed in many cancer types indicating it may be involved in carcinogenesis and tumour progression [133-136]. E2F2, a transcription factor involved in cell cycle progression was
also chosen. It is also directly regulated by Myc through activation of an E-box element in its promoter [137]. Both these genes would be expected to be down-regulated in response to a decreased Myc level.

A third direct Myc target, CDKN1A, is a Cyclin-dependent kinase inhibitor (p21) which can bind a variety of cyclin-dependent kinases, functioning as a regulator of cell cycle progression and mediating cellular senescence [138]. Myc directly interacts with the CDKN1A promoter via recruitment by the DNA binding protein Miz-1, blocking activation by p53, p73 and other activators and inhibiting transcription [139, 140]. Myc inhibition would therefore be predicted to result in an increase in transcription of this gene.

The fourth gene chosen, CCND1, encodes Cyclin D1, a regulatory subunit of the cyclin-dependent kinases CDK4 and CDK6, which are required for progression through the G1 phase of the cell cycle [141]. There is evidence that Myc can repress transcription of CCND1 [142]. Myc inhibition would therefore be expected to down-regulate CCND1 expression. Fibroblasts lacking D-type cyclins 1-3 are unable to be transformed by Myc suggesting an important role for D-type cyclins in Myc-mediated transformation [143].

As well as downstream targets, Myc mRNA levels were also quantified. Myc has a negative autoregulation mechanism resulting in transcription being repressed in response to Myc-Max heterodimers [144]. Myc sequestration should result in less Myc-Max heterodimers and a predicted increase in Myc mRNA levels.
5.5.2 qRT-PCR confirms RNA-seq data on selected downstream Myc targets in 1746-Omomyc treated cells

T11 cells were treated with 1746-Omomyc, Penetratin-Omi, Mutant Penetratin-Omi and controls and qRT-PCR was carried out using probes for the selected Myc target genes. The results from the 1746-Omomyc treated cells confirmed the data from the RNA-Seq analysis and is consistent with Myc inhibition. The direction of regulation was the same for all genes tested; however all genes were either up or down-regulated to a greater extent in the RNA-Seq analysis than was seen with qRT-PCR (Table 6). Myc and CDKN1A mRNA levels were substantially upregulated to 1.63 and 4.87 fold compared to the control cells, while CCND1, MINA and E2F2 levels were substantially down-regulated with 0.34, 0.39 and 0.70 fold expression compare to the control (Figure 10).

Table 6: Comparison of RNA-Seq and qRT-PCR expression data on Myc targets in T11 cells treated with 1746-Omomyc at 5µM for 3 hours. Data are fold change relative to untreated controls.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RNA-seq (fold change)</th>
<th>qRT-PCR (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc</td>
<td>2.59</td>
<td>1.63</td>
</tr>
<tr>
<td>Cdkn1A</td>
<td>8.03</td>
<td>4.87</td>
</tr>
<tr>
<td>MINA</td>
<td>0.34</td>
<td>0.39</td>
</tr>
<tr>
<td>E2F2</td>
<td>0.39</td>
<td>0.70</td>
</tr>
<tr>
<td>CCND1</td>
<td>0.19</td>
<td>0.34</td>
</tr>
</tbody>
</table>

5.5.3 Expression of Myc targets consistent with Myc inhibition

The regulation of Myc targets was consistent with Myc inhibition with up-regulation of Myc and CDKN1A, and down-regulation of MINA and CCND1 in response to 1746-Omomyc, Penetratin-Omi and Mutant Penetratin-Omi (Figure 10). E2F2, however was only downregulated in response to 1746-Omomyc and not the Penetratin conjugates.
Although Myc levels were higher in the Mutant Penetratin-Omi treated cells, the responses of the other Myc target genes were similar for Penetratin-Omi and Mutant Penetratin-Omi treated cells.

\[
\text{Myc was upregulated to 2.92 fold in Penetratin-Omi and 4.70 fold in Mutant Penetratin-Omi treated cells. CDKN1A was substantially upregulated to 3.61 fold for Penetratin-Omi and 3.39 fold for the Mutant treated cells. MINA was down-regulated to 0.48 fold for Penetratin-Omi and 0.50 fold for the mutant. CCND1A was also down-regulated to 0.70 fold for Penetratin-Omi and 0.61 fold for the Mutant. E2F2 was not substantially downregulated as was seen in the 1746 and 1746-Omomyt treated cells but remained close to what was expressed in the control with 1.10 fold expression in Penetratin-Omi and 1.00 fold expression in the Mutant treated cells. The direction of regulation of four of the genes was the same in the Penetratin-Omi and Mutant Penetratin-Omi treated cells.}
\]

**Figure 10**: mRNA levels of selected downstream Myc targets determined by qRT-PCR in T11 cells. Cells were treated with 1746-Omomyc, 1746 and Omomyc at 5µM and Penetratin-Omi and Mutant Penetratin-Omi at 50µM for 3 hours. Calculated using the Delta Delta Ct method with GAPDH as the reference gene and normalised to expression levels in untreated cells. Error bars represent SEM from a single experiment.
as was seen for 1746-Omomyc (all except E2F2). With the exception of Myc the changes were substantially greater in the 1746-Omomyc treated cells however.

The expression of E2F2 was very similar in 1746-Omomyc and 1746 treated cells (0.70 and 0.66 Fold) and was not downregulated in the Penetratin-Omi and Mutant Penetratin-Omi treated cells. 1746 treated cells showed elevated Myc expression (1.21 fold) and CDKN1A expression (1.79 fold).
6. Discussion

These results reveal the use of 1746-Omomyc as a promising strategy for inhibition of Myc in TNBC. It inhibited the growth of several TNBC cell lines at reasonably low concentrations via a reduction in cellular proliferation. The results were consistent with other studies utilising Omomyc in which a marked reduction in proliferation was seen [6, 145]. Omomyc shows great potential as a Myc inhibitor and anti-cancer agent. It has several benefits that come with using a protein based inhibitor, including a potentially high affinity and specificity for its target and low potential for toxicity. This work shows that the 1746 CPP was successful at delivering Omomyc into the cell while maintaining its therapeutic activity. The CPP-peptide conjugates also displayed some selectivity toward cancer cells over non-cancerous cell lines; a property which may make them useful for the delivery of other cancer targeted therapies and worthy of further investigation. A significant loss of activity and change in mechanism of action was seen with the truncated peptide conjugates. Further investigation into their design is likely to be beneficial in improving their activity and reducing the concentrations required.

6.1 Myc levels in cell panels

In both the murine and human cell lines tested the highest Myc expression was seen in a cell line of the claudin-low subtype. Although the Myc levels in the basal-like cell lines were not much lower in the murine panel; in the human panel the SUM159 cells had substantially higher levels of Myc. This was in contrast to other studies which have characterised claudin-low tumours as typically having lower Myc expression than the basal-like subtype [146].
The increased expression of Myc in T11 cells was modest at only 1.35 fold the expression seen in the NIH-3T3 cells. As NIH-3T3s are an immortalised cell line it should be noted that there is a possibility that it may also overexpress Myc. It must also be considered that mRNA levels often do not correlate with protein level. Post-transcriptional processes contribute greatly to the final amount of protein present and can work in amplification or competition with transcriptional signals [147]. It would therefore be advantageous to also quantify the level of the Myc protein in each cell line.

The T11 cell line is deficient in the tumour suppressor p53. p53 is mutated in a large proportion of human breast cancers and is especially prominent in tumours of the basal-like subtype and in BRCA1-mutated tumours [148]. The p53 mutation also acts as a marker indicative of poor prognosis and chemotherapy resistance [149]. Gene expression analyses suggest that this model may mimic human tumours more closely than many other models [18, 150]. For these reasons, T11 cells were chosen as a model for subsequent experiments.

6.2 Variability in response to 1746-Omomyx

A correlation of the response to the 1746-Omomyx peptide and the Myc mRNA level in the human panel was found. This analysis was based on very limited experimental results as Myc levels were only quantified once for each cell line and only a small number of cell lines were tested. This could be improved by adding more cell lines and replicating the results. An R$^2$ value of 0.718 (SE 0.230) for this model indicates that a substantial proportion of the difference in response to the peptide may be accounted for by the cells’ Myc levels, but also that there are other factors that are significantly contributing to the
variability in the response to this treatment. These could include cell division time, tumour subtype and membrane composition.

The cell division time of each cell line was not considered and results were read after a fixed time of 24 hours. Slower dividing cell lines may be responding well to the treatment, but this may not equate to a large percentage of inhibition compared to the controls in only 24 hours. This may be a confounding factor that should be considered in future studies. It would be useful to compare cell lines with similar cell division times, but large differences in Myc levels.

It is also of interest whether there is any correlation between the response to the treatment and the tumour subtype. In the human panel there does appear to be a correlation of response to the 1746-Omomyc peptide and the breast cancer subtype with claudin-low and basal-like tumours showing the greatest response and the luminal tumours a lesser response. More data would be needed to draw any definitive conclusions. This trend doesn’t appear to be based entirely on the level of Myc expressed in the cells. For example, the response to the peptide was significantly different for the luminal ZR-75-1 cells and the basal-like SUM149 cells although the Myc levels measured were similar. The complexity of Myc regulation and the heterogeneity of breast cancer leave a large window for differences in response to a Myc inhibitor. Some cancer types may have a genetic program making them more dependent on Myc amplification than other types for proliferation. This may be more likely in basal-like tumours as a core Myc gene expression signature has been found to be more prominent in basal-like cancers than in other cancer types [3]. Given Myc’s integral role in stem cell biology and the enrichment
of stem-cell like features in claudin-low tumours, it is possible that the claudin-low cell lines tested were also highly dependent on Myc amplification for proliferation.

As the uptake of CPPs is dependent on electrostatic interactions, the membrane composition of each cancer cell population is likely also a driving factor in the variability of response to 1746-Omomyc. Cells with more negatively charged components on the cell membrane may favour the uptake of the positively charged peptide.

### 6.3 Mechanism of action

A reduction in cell viability was not seen in T11 cells when treated with 1746 or Omomyc alone. This was expected as the unconjugated Omomyc peptide is unable to penetrate the cell unaided. Cell viability was greatly reduced however when treated with the 1746-Omomyc conjugate. This suggests that 1746-Omomyc is being successfully delivered into the cells. The lack of response to the free 1746 CPP suggests that the inhibition of the cells, when treated with the 1746-Omomyc peptide, is due to the cargo (Omomyc) rather than the CPP itself.

The results of the Ki-67 and Annexin V and Propidium Iodide assays taken together indicate that the reduction in the number of viable T11 cells treated with the 1746-Omomyc peptide is caused primarily by a reduction in the number of proliferating cells, rather than through cell death. In contrast, the results of these assays indicate that the primary cause of the reduction in the number of viable cells after treatment with the Penetratin-Omi and Mutant Penetratin-Omi peptides is through cell death rather than through reduced proliferation.
The primary mechanism of action of 1746-Omomyc (reduced proliferation) is in accord with the mechanism of action of Omomyc in which reduced proliferation and cell cycle arrest have been reported [6, 145]. Other studies on Omomyc have reported its ability to induce cell death via apoptosis [95, 145]. The mechanism of cell death (apoptosis or necrotic pathways) could not be determined from the Annexin V/PI assay as it was only carried out after treatment at a single time point but could be determined by tracking the changes in each population of cells over multiple time points.

6.4 Increased IC₅₀ for 1746-Omi

The protein-protein interaction between Myc and Omomyc is dependent on the proteins’ secondary structure. The average length of a helical domain in a protein is small, spanning only two to three helical turns (eight to twelve residues), suggesting the possibility of developing short but biologically relevant alpha helical peptides [151]. The organisation into the helical structure is energetically demanding however [152]. Once a peptide is excised from a parent protein they often adopt an ensemble of shapes reducing their ability to specifically bind their intended target and increasing their susceptibility to proteases [153].

The destabilisation of conformation in the case of the shorter peptide is one possible explanation for the great reduction in potency between 1746-Omomyc and 1746-Omi. Several strategies have been developed for the stabilisation of short peptide sequences into helices. These include non-natural amino acid substitutions, helix capping, side chain constraints and hydrogen bond surrogates [154-157]. One or more of these strategies may be useful in improving the stabilisation of the truncated peptide.
Another factor that may have contributed to the decreased activity in the shorter CPP conjugates is the lack of a linker between the CPP and cargo domains. The 1746-Omomyc peptide contains a short 3 amino-acid linker (GAS). It has been shown that the absence of a suitable linker can lead to protein misfolding or impaired biological activity [158-160]. Most natural multi-domain proteins have a linker sequence with the average length being 6.5 amino acids long [161]. The investigation and inclusion of a suitable linker in the shorter CPP conjugates could be beneficial in improving the biological activity of the peptides.

6.5 Greater response for Penetratin-Omi than 1746-Omi

Penetratin-Omi was found to have greater activity than 1746-Omi at a range of concentrations tested. This could be due to the reduced internalisation of the peptide or decreased biological activity once inside the cell. There are several factors that may contribute to differences in internalisation including the peptides size, charge, secondary structure and amino acid composition.

Penetratin-Omi is smaller in size than 1746-Omi, with 44 amino acids compared to 66 amino acids, respectively. Although an increased size may affect penetrability, there are other factors that are likely more important. Due to the negative charge of cell membranes the charge of a CPP is an important factor in internalisation. 1746-Omi has a greater net positive charge at 17.1 than Penetratin-Omi at 7.1. However, the greater charge did not lead to greater activity in this case. There are other differences between the peptides that may affect their internalisation or activity. 1746 is lysine rich whilst Penetratin also contains critical arginine residues. This may cause differences in the mechanism of membrane binding with the contribution from hydrogen bonding especially prominent in
oligoarginine based CPPs compared to polylysine-based ones [109]. CPPs are often classified based on their amphipathicity as this also plays an important role in the mechanism of CPP membrane binding. Strong hydrophobic interactions are observed for amphipathic, but not for cationic CPPs [109]. Penetratin is considered a secondary amphipathic peptide as it contains a hydrophobic side and a cationic side only when in an alpha-helical conformation. Penetratin contains several hydrophobic amino acids, having a hydrophobicity score at pH 6.8 of 23. 1746 has quite different properties; is a cationic CPP made up almost entirely of hydrophilic amino acids with a hydrophobicity score at pH 6.8 of -22.74. Some of the properties of the CPPs are summarised in Table 7.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
<th>Net Charge</th>
<th>Hydrophobicity</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1746</td>
<td>Sequence unable to be disclosed</td>
<td>17.0</td>
<td>-22.74</td>
<td>4.538</td>
</tr>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRRMKWKK</td>
<td>7.0</td>
<td>23.00</td>
<td>2.247</td>
</tr>
</tbody>
</table>

To calculate net charge acidic amino acid residues are assigned a value of 1, basic amino acid residues -1 and neutral amino acid residues 0. Hydrophobicity at pH 6.8 calculated using BioSynthesis Peptide Property Calculator version 3.1

Investigating the level of peptide internalisation would also be useful in determining if the difference in activity between the two peptides was due to a lower biological activity or a reduced internalisation efficiency. This can be challenging however, as studies have shown that the conjugation of a fluorophore or ligand often produces confounding effects that may alter the internalisation, toxicity or functioning of a CPP [162, 163]. This was seen with the FITC labelled 1746 CPP. The increased toxicity demonstrates the difficulty faced in easily assessing internalisation of CPPs and indicates that FITC labelling is an unviable option in this case.
6.6 Cancer Selectivity

Both 1746-Omi and Penetratin-Omi showed complete selectivity towards the tumorigenic T11 cells over the non-tumorigenic NIH-3T3 cell line which showed no decrease in cell viability even at a concentration of 50µM. This may be partially attributed to differences in the charge of the cells. Due to changes in metabolism, cancer cells are known to have a more negative surface charge than non-tumorigenic cells [164, 165]. Since both peptides are positively charged, electrostatic interactions would favour the interaction of these peptides with the tumorigenic cells. This result was consistent with other studies in which other alpha-helical membrane-active proteins have shown specificity towards cancer cells [166]. Interestingly, this cancer cell selectivity was not as strong with 1746-Omomyc, despite 1746-Omomyc also having a large positive net charge, with some reduction in cell viability evident in NIH-3T3 cells at only 1µM. The hydrophobicity of alpha-helical peptides has also been shown to be an important factor in membrane interactions with anti-cancer activity being correlated with peptide hydrophobicity [166].

6.7 Mutant Penetratin-Omi

In the Mutant Penetratin-Omi peptide, alanine was used to replace the four amino acids that are critical in Omomyc’s binding to Myc. Alanine was chosen as it is chemically inert being non-polar and having a non-bulky methyl functional group that is non-reactive and rarely involved in protein function [167]. Alanine also has a high propensity for helix formation and would not be expected to hinder the formation of the peptides secondary structure [168]. Interestingly, the Mutant Penetratin-Omi also significantly reduced cell viability in T11 cells. There are several possibilities that could explain this result. It is possible that the changes introduced were not sufficient to stop the peptide interacting
with Myc. It may be more suitable to use charged amino acids for some of the critical substitutions.

### 6.8 Cargo dependent Cytotoxicity?

Another possibility is that Penetratin-Omi and Mutant peptides are wholly or partially reducing cell viability through another mechanism that is not based on Myc inhibition. The results of the proliferation and Apoptosis/Dead Cell Assays confirm that this is likely occurring to some extent because the primary mechanism of reduced cell viability was not through reduced proliferation as was seen for the 1746-Omomyc peptide but rather through cell death. It is possible that a cargo-dependent cytotoxicity of the CPP-conjugates is occurring at the higher concentrations tested. There was no reduction in cell viability when treated with free Penetratin up to the maximum concentration tested of 50µM. This is in accord with other studies. Penetratin is generally thought to be non-toxic and has been shown not to significantly decrease cell viability even at concentrations up to 100µM [169]. However, the cytotoxicity of a CPP has been shown to vary depending on its context and is highly dependent on the attached cargo [162]. Cargo dependent cytotoxicities have been reported for Penetratin-peptide conjugates. This can be seen in a study of the effect of a peptide targeting pancreatic cancer cells in which addition of the penetratin sequence was shown to be responsible for a change in the mechanism of action, inducing necrosis rather than apoptosis, which was observed in Penetratin’s absence [170]. Due to the complexity of factors at play it is difficult to make comparisons between one study and another.

To further investigate if this effect is due to the cargo or the CPP itself, it would be useful to also determine and compare the mechanism of reduced cell viability for 1746-Omi. It
would be beneficial to include it in any future experiments. The Annexin V/Propidium Iodide assays should be repeated using multiple time points to gain a clearer understanding of whether the cell death was occurring primarily through apoptotic or necrotic pathways.

6.9 Myc Inhibition

Although the qRT-PCR analyses of the downstream Myc targets was able to confirm the results of the RNA-seq analysis for the selected genes, a full analysis of the Myc pathways from the RNA-seq data is needed to confirm Myc inhibition. Similarly, the qRT-PCR results on the Penetratin-Omi and Mutant Penetratin-Omi peptides suggest that a level of Myc inhibition may be occurring in response to the peptides, but RNA-seq would be needed to confirm this.

The expression of the selected targets were affected similarly for Penetratin-Omi and Mutant Penetratin-Omi treated cells. This suggests they these two peptides are likely both working through the same mechanism of action. The same pattern of expression of the Myc targets was seen for the penetratin conjugated peptides as was seen for 1746-Omomyc, although lower in magnitude (excluding Myc). This suggests that the mechanism of action of the Penetratin-Omi and Mutant Penetratin-Omi may in part be similar to the mechanism of the 1746-Omomyc peptide but with a lower level of activity.

The similar expression of E2F2 in 1746-Omomyc and 1746 treated cells and lack of downregulation in the Penetratin-Omi and Mutant Penetratin-Omi treated cell indicate that the response in this gene could be due to the CPP itself.
This data leaves open the possibility of a degree of Myc inhibition in response to both the Penetratin-Omi and Mut Penetratin-Omi, but does not confirm this. It would be of great value to include Penetratin as a control to rule out the changes caused by the CPP itself. As the experiment was only carried out a single time a statistical analysis of the differences between treatments is not possible.

The upregulation of Myc transcription in response to the peptides was of interest as the negative autoregulation of the Myc gene has been shown to require the Myc-Max heterodimer [144]. This suggests that the interference of the formation of these dimers may have been successful. This result fits in with other studies which have confirmed that Myc expression is downregulated in response to the Myc protein [171]. There are, however, other critical pathways responsible for Myc regulation whose influence cannot be ruled out.

CDKN1A was greatly upregulated in response to 1746-Omomyc as well as the penetratin-conjugated peptides suggesting successful Myc inhibition. There are a variety of signals and factors that are involved in the transcriptional regulation of CDKN1A including the tumour suppresser p53 [172]. Some studies have reported the requirement of an intact p53 pathway for the treatment of cancer via Myc targeting [173]. This is of interest as the T11 cell line used in this study is a p53 null model. Interestingly, the induction of G1 arrest by Omomyc has been found to be dependent on the activation of CDKN1A even in cancer cells with genetic TP53 inactivation [145]. This is thought to occur due to Myc’s disruption of p73 mediated transcriptional activation of the CDKN1A gene.
Interestingly, 1746 treated cells also showed a degree of increased CDKN1A expression. This indicates that some of the increase in expression may also be attributable to a mechanism other than Myc inhibition. A degree of Myc-independent regulation is likely as this gene has been shown to be upregulated in response to a variety of stimuli [174]. This could be caused by the CPP itself or the entry of the peptide into the cell.

The difference in expression in the other three genes was not as pronounced as was seen with Myc and CDKN1A. This is typical of gene expression studies involving Myc in which the average effect on expression of target genes is modest.

6.10 Conclusions and Future Directions

1746-Omomyc was successful in reducing cancer cell viability in triple-negative breast cancer cell lines overexpressing Myc at reasonably low concentrations. There was also some evidence of cancer selectivity with non-tumorigenic cell lines having a greatly reduced response to the peptide. This initial exploration of the mechanisms involved indicates that the reduction in cell viability is through reduced cellular proliferation rather than cell death and is consistent with Myc inhibition. Whether the cell death that is occurring is due to apoptotic or necrotic pathways could be further evaluated by repeating the Annexin V/PI Assay at multiple time points.

A much higher concentration of the truncated version of the peptides (1746-Omi and Penetratin-Omi) was required to reduce cancer cell viability indicating that the biological activity of the cargo was greatly reduced. It may be beneficial to deduce the level of biological activity of the Omi peptide via a different method before adding the increased
complexity of the effects of the CPPs themselves. This could be done by establishing a cell line with inducible Omi expression.

There was evidence of cancer cell selectivity of Penetratin-Omi and 1746-Omi. If Omi is found to be biologically relevant, improvements in the design of the CPP conjugated peptides may increase the activity of the cargo. This could include further investigation into design mechanisms that aid in alpha helix stabilisation and the use of suitable linkers between the peptides two domains.

A mutant version of the Penetratin-Omi peptide with critical amino acids involved in dimerization altered was also found to reduce cell viability, although not as significantly as the unaltered peptide. The primary mechanism of action of both peptides was found to be through cell death rather than reduced proliferation as was seen for 1746-Omomyc. Further investigation into the design of the mutant peptide would be useful to ensure it is not interacting with Myc. The possibility of a cargo dependent cytotoxicity occurring at the high concentrations that were needed to significantly reduce cell viability was uncovered. Increasing the activity of the peptide through the measures discussed above could reduce the peptide concentration required, reducing the chance of any potential cytotoxic side effects. Penetratin-Omi reduced cell viability in T11 cells significantly more than 1746-Omi at several concentrations tested. Due to the possibility of Penetratin-Omi producing a cargo-dependent cytotoxicity, it would be useful to also determine the mechanism of action of the 1746-Omi peptide.

qRT-PCR confirmed the results of an RNA-seq analysis on selected downstream Myc targets in 1746-Omomyc treated cells. The regulation of downstream Myc targets was
consistent with, but cannot confirm Myc inhibition in 1746-Omomyc, Penetratin-Omi and Mutant Penetratin-Omi treated cells. Myc inhibition could be confirmed via analysis of all Myc pathway genes using RNA-seq.

Although more work is needed, the novel approach to Myc inhibition utilised in this study looks to be a promising strategy that may eventually assist in the treatment of TNBCs.
7. References


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8. Supplementary Figures

Supplementary Figure 1: Dose response data of 1746 and FITC-1746 in T11 cells treated at concentrations from 0.25 to 50µM for 24 hours and cell viability assessed using CellTiter-Glo Assays. Percentage of viable cells was normalised to non-treated control cells. All data points are presented as mean ± SEM for three independent experiments for 1746 and two independent experiments for FITC-1746.

Supplementary Figure 2: Controls form Annexin V and Propicium Iodide Assay. FACS analysis of T11 cells treated for 24 hours with 1746 at 2.5µM, Omomyc at 2.5µM, Penetratin at 25µM and no treatment (control) stained with FITC-Annexin V and Propidium Iodide.